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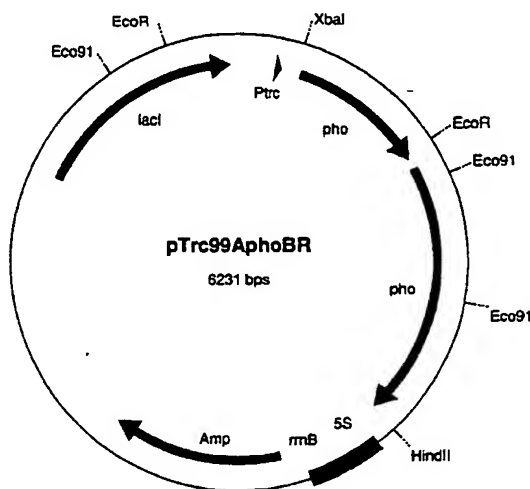
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(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY WHICH CONTAIN AN ENHANCED PHOB OR PHOR GENE



(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which at least one or more of the genes chosen from the group consisting of phoB and phoR, or nucleotide sequences which code for these, is or are enhanced, in particular over-expressed, b) concentration of the desired L-amino acid in the medium or in the cells of the bacteria, and c) isolation of the desired L-amino acid.

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**Process for the Preparation of L-Amino Acids using  
Strains of the Enterobacteriaceae Family which Contain  
an Enhanced *phoB* or *phoR* Gene**

Field of the Invention

- 5 This invention relates to a process for the preparation of L-amino acids, in particular L-threonine, using strains of the Enterobacteriaceae family in which at least one or more of the genes chosen from the group consisting of *phoB* and *phoR* is (are) enhanced.
- 10 Prior Art
- L-Amino acids, in particular L-threonine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.
- 15 It is known to prepare L-amino acids by fermentation of strains of Enterobacteriaceae, in particular *Escherichia coli* (*E. coli*) and *Serratia marcescens*. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the
- 20 process can relate to fermentation measures, such as e.g. stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form, by e.g. ion exchange chromatography, or the intrinsic output
- 25 properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue  $\alpha$ -

30 amino- $\beta$ -hydroxyvaleric acid (AHV), or are auxotrophic for metabolites of regulatory importance and produce L-amino acid, such as e.g. L-threonine are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production.

#### Object of the Invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

#### Summary of the Invention

The invention provides a process for the fermentative preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in particular already produce L-amino acids and in which at least one or more of the nucleotide sequence(s) which code(s) for the phoB and phoR genes is (are) enhanced.

#### Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene or allele which codes for a corresponding enzyme or

protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is  
5 in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

10 The process comprises carrying out the following steps:

- a) fermentation of microorganisms of the Enterobacteriaceae family in which one or more of the genes chosen from the group consisting of phoB and phoR, or nucleotide sequences which code for  
15 these, is enhanced, in particular over-expressed,
- b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and
- c) isolation of the desired L-amino acid, constituents  
20 of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.

The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose,  
25 fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are  
30 preferred. Of the genus Escherichia the species Escherichia coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.

Suitable strains, which produce L-threonine in particular, of the genus *Escherichia*, in particular of the species *Escherichia coli*, are, for example

- Escherichia coli TF427
- 5 Escherichia coli H4578
- Escherichia coli KY10935
- Escherichia coli VNIIGenetika MG442
- Escherichia coli VNIIGenetika M1
- Escherichia coli VNIIGenetika 472T23
- 10 Escherichia coli BKIIM B-3996
- Escherichia coli kat 13
- Escherichia coli KCCM-10132.

Suitable L-threonine-producing strains of the genus *Serratia*, in particular of the species *Serratia marcescens*,  
15 are, for example

- Serratia marcescens* HNr21
- Serratia marcescens* TLr156
- Serratia marcescens* T2000.

Strains from the Enterobacteriaceae family which produce L-  
20 threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of: resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to  $\alpha$ -methylserine, resistance to diaminosuccinic acid,  
25 resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate, resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a  
30 partial and compensable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid,

resistance to L-aspartate, resistance to L-leucine,  
resistance to L-phenylalanine, resistance to L-serine,  
resistance to L-cysteine, resistance to L-valine,  
sensitivity to fluoropyruvate, defective threonine  
5 dehydrogenase, optionally an ability for sucrose  
utilization, enhancement of the threonine operon,  
enhancement of homoserine dehydrogenase I-aspartate kinase  
I, preferably of the feed back resistant form, enhancement  
of homoserine kinase, enhancement of threonine synthase,  
10 enhancement of aspartate kinase, optionally of the feed  
back resistant form, enhancement of aspartate semialdehyde  
dehydrogenase, enhancement of phosphoenol pyruvate  
carboxylase, optionally of the feed back resistant form,  
enhancement of phosphoenol pyruvate synthase, enhancement  
15 of transhydrogenase, enhancement of the RhtB gene product,  
enhancement of the RhtC gene product, enhancement of the  
YfiK gene product, enhancement of a pyruvate carboxylase,  
and attenuation of acetic acid formation.

It has been found that microorganisms of the  
20 Enterobacteriaceae family produce L-amino acids, in  
particular L-threonine, in an improved manner after  
enhancement, in particular over-expression, of at least one  
or more of the genes chosen from the group consisting of  
phoB and phoR.

25 The nucleotide sequences of the genes of Escherichia coli  
belong to the prior art and can also be found in the genome  
sequence of Escherichia coli published by Blattner et al.  
(Science 277: 1453-1462 (1997)).

The following information, inter alia, on the phoB and phoR  
30 genes is known from the prior art:

phoB gene:

Description: Regulatory protein of the pho regulon

Reference: Makino et al.; Journal of Molecular Biology  
190(1): 37-44 (1986); Mc Cleary; Molecular  
Microbiology 20(6): 1155-1163 (1996);  
Torriani; Bioessays 12(8): 371-376 (1990)

5 Accession No.: AE000146

Alternative gene names: phoRc, phoT

phoR gene:

Description: Sensor protein of the pho regulon

EC No.: 2.7.3.-

10 Reference: Makino et al.; Journal of Molecular Biology  
192(3): 549-556 (1986); Torriani; Bioessays  
12(8): 371-376 (1990); Yamada et al.;  
Molecular and General Genetics 220(3): 366-  
372 (1990)

15 Accession No.: AE000146

Alternative gene names: Rlpho, nmpB, phoR1

The nucleic acid sequences can be found in the databanks of  
the National Center for Biotechnology Information (NCBI) of  
the National Library of Medicine (Bethesda, MD, USA), the  
20 nucleotide sequence databank of the European Molecular  
Biologies Laboratories (EMBL, Heidelberg, Germany or  
Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima,  
Japan).

The genes described in the text references mentioned can be  
25 used according to the invention. Alleles of the genes which  
result from the degeneracy of the genetic code or due to  
"sense mutations" of neutral function can furthermore be  
used.

To achieve an enhancement, for example, expression of the  
30 genes or the catalytic properties of the proteins can be  
increased. The two measures can optionally be combined.

To achieve an over-expression, the number of copies of the  
corresponding genes can be increased, or the promoter and

regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is  
5 additionally possible to increase the expression in the course of fermentative L-threonine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme  
10 protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of  
15 the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), in Hartley and Gregori (Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40: 183-190  
20 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80: 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and Klipp (Gene 80: 161-169  
25 (1989)), in Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)) and in known textbooks of genetics and molecular biology.

Plasmid vectors which are capable of replication in  
30 Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)), pTrc99A (Amann et al.; (Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia; Proceedings of the National Academy of Sciences of the United States of America 80  
35 (21): 6557-6561 (1983)) can be used. A strain transformed



with a plasmid vector, where the plasmid vector carries at least one or more of the genes chosen from the group consisting of phoB and phoR, or nucleotide sequences which code for these, can be employed in a process according to  
5 the invention.

It is also possible to transfer mutations which affect the expression of the particular gene into various strains by sequence exchange (Hamilton et al.; Journal of Bacteriology 171: 4617-4622 (1989)), conjugation or transduction.

- 10 It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family, in addition to enhancement of one or more of the genes chosen from the group consisting of phoB and phoR, for one or more enzymes of the known  
15 threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate or enzymes of glycolysis or PTS enzymes or enzymes of sulfur metabolism to be enhanced.
- 20 Thus, for example, at the same time one or more of the genes chosen from the group consisting of
  - the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
  - 25 • the pyc gene of Corynebacterium glutamicum which codes for pyruvate carboxylase (WO 99/18228),
  - the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231(2): 332-336 (1992)),
  - 30 • the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),

- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- 5 • the mgo gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1 013 765),
- the thrE gene of Corynebacterium glutamicum which codes  
10 for the threonine export protein (WO 01/92545),
- the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),
- the hns gene which codes for the DNA-binding protein  
15 HLP-II (Molecular and General Genetics 212: 199-202 (1988)),
- the pgm gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994)),
- the fba gene which codes for fructose biphosphate  
20 aldolase (Biochemical Journal 257: 529-534 (1989)),
- the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- 25 • the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase

- system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the ptsG gene which codes for the glucose-specific IIBC component (Journal of Biological Chemistry 261: 16398-16403 (1986)),
  - the lrp gene which codes for the regulator of the leucine regulon (Journal of Biological Chemistry 266: 10768-10774 (1991)),
  - the mopB gene which codes for 10 Kd chaperone (Journal of Biological Chemistry 261: 12414-12419 (1986)) and is also known by the name groES,
  - the ahpC gene of the ahpCF operon which codes for the small sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences of the United States of America 92: 7617-7621 (1995)),
  - the ahpF gene of the ahpCF operon which codes for the large sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences of the United States of America 92: 7617-7621 (1995)),
  - the cysK gene which codes for cysteine synthase A (Journal of Bacteriology 170: 3150-3157 (1988)),
  - the cysB gene which codes for the regulator of the cys regulon (Journal of Biological Chemistry 262: 5999-6005 (1987)),
  - the cysJ gene of the cysJIH operon which codes for the flavoprotein of NADPH sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
  - the cysI gene of the cysJIH operon which codes for the haemoprotein of NADPH sulfite reductase (Journal of

- Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- the *cysH* gene of the *cysJHI* operon which codes for adenylyl sulfate reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
  - the *phoE* gene which codes for protein E of the outer cell membrane (Journal of Molecular Biology 163 (4): 513-532 (1983)),
  - 10 • the *malE* gene which codes for the periplasmic binding protein of maltose transport (Journal of Biological Chemistry 259 (16): 10606-10613 (1984)),
  - the *pykF* gene which codes for fructose-stimulated pyruvate kinase I (Journal of Bacteriology 177 (19): 5719-5722 (1995)),
  - 15 • the *pfkB* gene which codes for 6-phosphofructokinase II (Gene 28 (3): 337-342 (1984)),
  - the *talB* gene which codes for transaldolase B (Journal of Bacteriology 177 (20): 5930-5936 (1995)),
  - 20 • the *rseA* gene of the *rseABC* operon which codes for a membrane protein with anti- $\sigma^E$  activity (Molecular Microbiology 24 (2): 355-371 (1997)),
  - the *rseC* gene of the *rseABC* operon which codes for a global regulator of the  $\sigma^E$  factor (Molecular Microbiology 24 (2): 355-371 (1997)),
  - 25 • the *sodA* gene which codes for superoxide dismutase (Journal of Bacteriology 155 (3): 1078-1087 (1983)),
  - the *sucA* gene of the *sucABCD* operon which codes for the decarboxylase sub-unit of 2-ketoglutarate dehydrogenase

(European Journal of Biochemistry 141 (2): 351-359 (1984)),

- the sucB gene of the sucABCD operon which codes for the dihydrolipoyltranssuccinase E2 sub-unit of 2-ketoglutarate dehydrogenase (European Journal of Biochemistry 141 (2): 361-374 (1984)),
- the sucC gene of the sucABCD operon which codes for the  $\beta$ -sub-unit of succinyl-CoA synthetase (Biochemistry 24 (22): 6245-6252 (1985)) and
- 10 • the sucD gene of the sucABCD operon which codes for the  $\alpha$ -sub-unit of succinyl-CoA synthetase (Biochemistry 24 (22): 6245-6252 (1985)),

can be enhanced, in particular over-expressed.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to enhancement of one or more of the genes chosen from the group consisting of phoB and phoR, for one or more of the genes chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- the gene product of the open reading frame (orf) yjfa (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),

- the pckA gene which codes for the enzyme phosphoenol pyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- 5 • the poxB gene which codes for pyruvate oxidase (Nucleic Acids Research 14(13): 5449-5460 (1986)),
- the aceA gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- 10 • the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (Bioscience, Biotechnology and Biochemistry 59: 256-251 (1995)) and is also known under the name of the mlc gene,
- the fruR gene which codes for the fructose repressor (Molecular and General Genetics 226: 332-336 (1991)) and is also known under the name of the cra gene and
- 15 • the rpoS gene which codes for the sigma<sup>38</sup> factor (WO 01/05939) and is also known under the name of the katF gene,

to be attenuated, in particular eliminated or for the expression thereof to be reduced.

- 20 The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a
- 25 corresponding enzyme with a low activity or inactivates the corresponding enzyme (protein) or gene, and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to

30 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of

the activity or concentration of the protein in the starting microorganism.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to  
5 enhancement of one or more of the genes chosen from the group consisting of *phoB* and *phoR*, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London,  
10 UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed batch process (feed process) or the repeated fed batch process (repetitive feed process). A summary of known  
15 culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und  
20 periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained  
25 in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally  
30 cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source

of carbon. These substances can be used individually or as a mixture.

- Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.
- 10 Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate,
- 15 which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to
- 20 the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

- Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed
- 25 in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of
- 30 plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino



acids or L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin  
5 derivation, as described by Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)), or it can take place by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the  
10 fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

15 The minimal (M9) and complete media (LB) for Escherichia coli used are described by J.H. Miller (A Short Course in Bacterial Genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, ligation, Klenow and  
20 alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). Unless described otherwise, the transformation of Escherichia coli is carried out by the method of Chung et al. (Proceedings  
25 of the National Academy of Sciences of the United States of America 86: 2172-2175 (1989)).

The incubation temperature for the preparation of strains and transformants is 37°C.

Example 1

Construction of the expression plasmid pTrc99AphoBR

The phoB and phoR genes from E. coli K12 are amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the phoB and phoR genes in E. coli K12 MG1655 (Accession Number AE000146, Blattner et al. (Science 277: 1453-1462 (1997))), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The sequences of the primers are modified such that recognition sites for restriction enzymes are formed. The recognition sequence for XbaI is chosen for the phoBR1 primer and the recognition sequence for HindIII for the phoBR2 primer, which are marked by underlining in the nucleotide sequence shown below:

phoBR1: 5' - CGCATTAATGATCGCAATCTAGATAGTACAAC - 3' (SEQ ID No. 1)

phoBR2: 5' - GGCTTATGGCAATAAAAGCTTACAAAGG - 3' (SEQ ID No. 2)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 2100 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated according to the manufacturer's instructions with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10. Selection of plasmid-carrying cells takes place on LB agar, to which 50 µg/ml kanamycin are added. After isolation of the plasmid DNA, the vector pCR-Blunt II-TOPO-phoBR is cleaved

with the restriction enzymes HindIII and XbaI and, after separation in 0.8% agarose gel, the phoBR fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligation is carried out with the phoBR fragment isolated. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes EcoRV and Eco91I. The plasmid is called pTrc99AphoBR (Figure 1).

#### Example 2

- 15 Preparation of L-threonine with the strain  
MG442/pTrc99AphoBR

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

- The strain MG442 is transformed with the expression plasmid pTrc99AphoBR described in example 1 and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 µg/ml ampicillin. The strains MG442/pTrc99AphoBR and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated

and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

250 µl portions of this preculture are transinoculated into 5 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot 1\text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in 10 the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

15 The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

20 The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AphoBR	5.7	2.7

Brief Description of the Figure:

Figure 1: Map of the plasmid pTrc99AphoBR containing the phoB and phoR genes.

The length data are to be understood as approx. data. The  
5 abbreviations and designations used have the following meaning:

- Amp: Ampicillin resistance gene
- lacI: Gene for the repressor protein of the trc promoter
- 10 • P<sub>trc</sub>: trc promoter region, IPTG-inducible
- phoB: Coding region of the phoB gene
- phoR: Coding region of the phoR gene
- 5S: 5S rRNA region
- rrnBT: rRNA terminator region
- 15 The abbreviations for the restriction enzymes have the following meaning
- EcoRV: Restriction endonuclease from Escherichia coli B946
- Eco91I: Restriction endonuclease from Escherichia coli  
20 RFL91
- HindIII: Restriction endonuclease from Haemophilus influenzae
- XbaI: Restriction endonuclease from Xanthomonas campestris

**What is claimed is:**

1. A process for the preparation of L-amino acids, in particular L-threonine, which comprises carrying out the following steps:
  - 5 a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which one or more of the genes chosen from the group consisting of phoB and phoR, or nucleotide sequences which code for these, is or  
10 are enhanced, in particular over-expressed,
  - b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
  - c) isolation of the desired L-amino acid, constituents  
15 of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100[sic]) thereof optionally remaining in the product.
2. A process as claimed in claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are  
20 employed.
3. A process as claimed in claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
- 25 4. A process as claimed in claim 1, wherein the expression of the polynucleotide (s) which code(s) for one or more of the genes chosen from the group consisting of phoB and phoR is increased.
- 30 5. A process as claimed in claim 1, wherein the regulatory and/or catalytic properties of the polypeptides

(proteins) for which the polynucleotides phoB and phoR code are improved or increased.

6. A process as claimed in claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
  - 6.2 the pyc gene which codes for pyruvate carboxylase,
  - 6.3 the pps gene which codes for phosphoenol pyruvate synthase,
  - 6.4 the ppc gene which codes for phosphoenol pyruvate carboxylase,
  - 6.5 the pntA and pntB genes which code for transhydrogenase,
  - 6.6 the rhtB gene which imparts homoserine resistance,
  - 6.7 the mqo gene which codes for malate:quinone oxidoreductase,
  - 6.8 the rhtC gene which imparts threonine resistance,
  - 6.9 the thrE gene which codes for the threonine export protein,
  - 6.10 the gdhA gene which codes for glutamate dehydrogenase,

- 6.11 the hns gene which codes for the DNA-binding protein HLP-II,
- 6.12 the pgm gene which codes for phosphoglucomutase,
- 5 6.13 the fba gene which codes for fructose biphosphate aldolase,
- 6.14 the ptsH gene which codes for the phosphohistidine protein hexose phosphotransferase,
- 10 6.15 the ptsI gene which codes for enzyme I of the phosphotransferase system,
- 6.16 the crr gene which codes for the glucose-specific IIA component,
- 6.17 the ptsG gene which codes for the glucose-specific IIBC component,
- 15 6.18 the lrp gene which codes for the regulator of the leucine regulon,
- 6.19 the mopB gene which codes for 10 Kd chaperone,
- 6.20 the ahpC gene which codes for the small sub-unit of alkyl hydroperoxide reductase,
- 20 6.21 the ahpF gene which codes for the large sub-unit of alkyl hydroperoxide reductase,
- 6.22 the cysK gene which codes for cysteine synthase A,
- 25 6.23 the cysB gene which codes for the regulator of the cys regulon,
- 6.24 the cysJ gene which codes for the flavoprotein of NADPH sulfite reductase,



- 6.25 the cysI gene which codes for the haemoprotein of NADPH sulfite reductase,
- 6.26 the cysH gene which codes for adenylyl sulfate reductase,
- 5 6.27 the phoE gene which codes for protein E of outer cell membrane,
- 6.28 the malE gene which codes for the periplasmic binding protein of maltose transport
- 10 6.29 the pykF gene which codes for fructose-stimulated pyruvate kinase I,
- 6.30 the pfkB gene which codes for 6-phosphofructokinase II,
- 6.31 the talB gene which codes for transaldolase B,
- 15 6.32 the rseA gene which codes for a membrane protein which acts as a negative regulator on sigmaE activity,
- 6.33 the rseC gene which codes for a global regulator of the sigmaE factor,
- 20 6.34 the sodA gene which codes for superoxide dismutase,
- 6.35 the sucA gene which codes for the decarboxylase sub-unit of 2-ketoglutarate dehydrogenase,
- 25 6.36 the sucB gene which codes for the dihydrolipoyltranssuccinase E2 sub-unit of 2-ketoglutarate dehydrogenase,
- 6.37 the sucC gene which codes for the  $\beta$ -sub-unit of succinyl-CoA synthetase,

6.38 the *sucD* gene which codes for the  $\alpha$ -sub-unit of succinyl-CoA synthetase,

is or are enhanced, in particular over-expressed, are fermented.

5 7. A process as claimed in claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:

10 7.1 the *tdh* gene which codes for threonine dehydrogenase,

7.2 the *mdh* gene which codes for malate dehydrogenase,

15 7.3 the gene product of the open reading frame (orf) *yjfa*,

7.4 the gene product of the open reading frame (orf) *ytfp*,

7.5 the *pckA* gene which codes for phosphoenolpyruvate carboxykinase,

20 7.6 the *poxB* gene which codes for pyruvate oxidase,

7.7 the *aceA* gene which codes for isocitrate lyase,

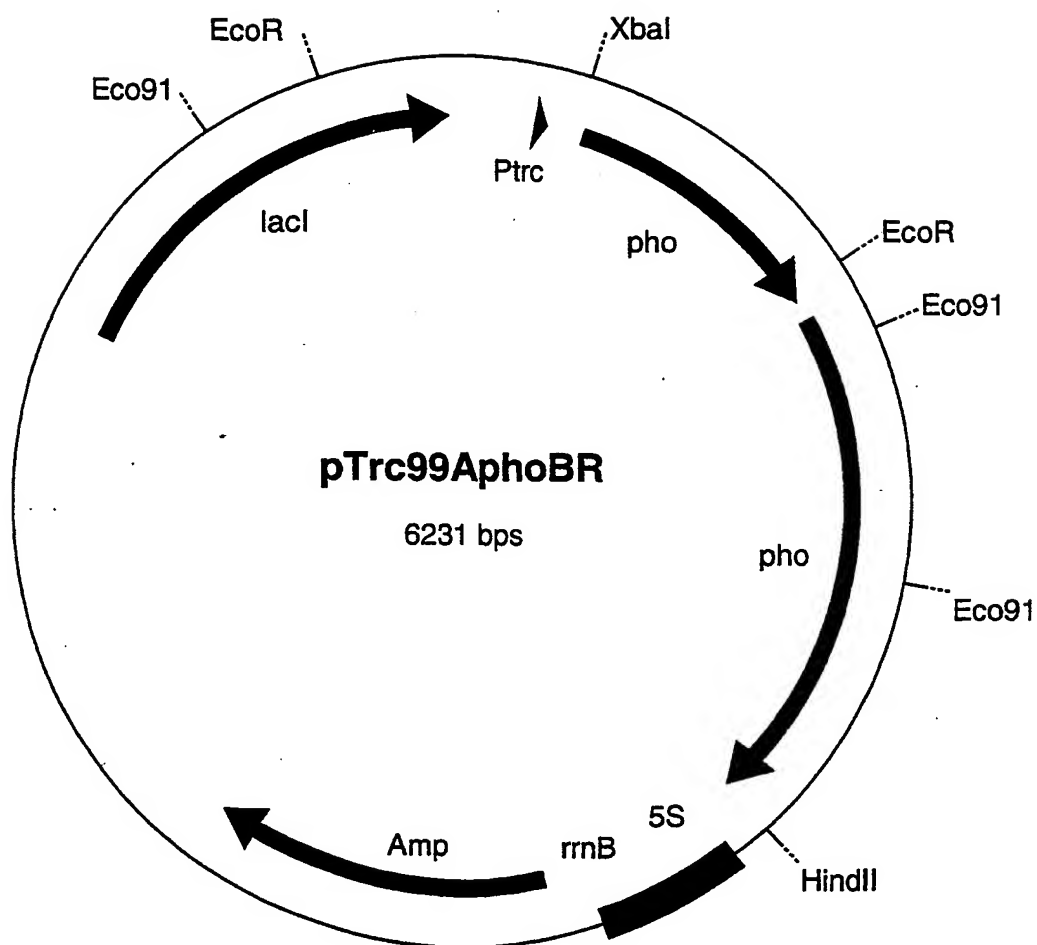
7.8 the *dgsA* gene which codes for the DgsA regulator of the phosphotransferase system,

25 7.9 the *fruR* gene which codes for the fructose repressor,

7.10 the *rpoS* gene which codes for the sigma<sup>38</sup> factor

is or are attenuated, in particular eliminated or reduced in expression, are fermented.

Fig. 1:



## SEQUENCE PROTOCOL

5 <110> Degussa AG  
<120> Process for the preparation of L-amino acids using  
strains of the Enterobacteriaceae family which contain an  
enhanced phoB or phoR gene  
<130> 020277BT  
<160> 2  
10 <170> PatentIn version 3.1  
<210> 1<211> 32<212> DNA<213> artificial sequence  
<220><221> Primer<222> (1)..(32)<223> phoBR1  
  
<400> 1  
15 cgcattaatg atcgcaatct agatagtaca ac  
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<210> 2<211> 28<212> DNA<213> artificial sequence  
<220><221> Primer<222> (1)..(28)<223> phoBR2  
20  
  
<400> 2  
ggcttatggc aataaaaagct tacaaaagg

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(75) Inventor/Applicant (for US only): **RIEPING, Mechthild**  
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LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
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European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,  
ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK,  
TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,  
GW, ML, MR, NE, SN, TD, TG).

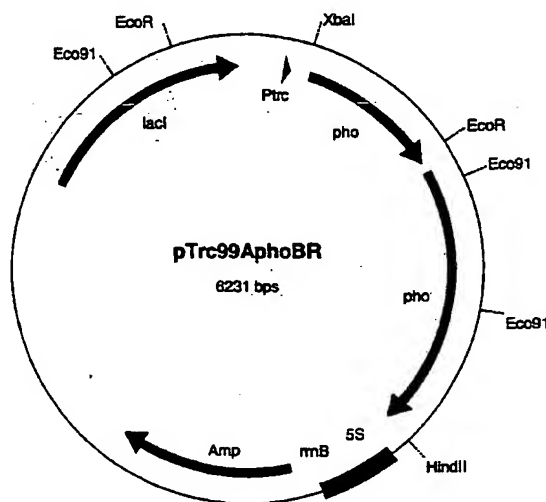
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For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE  
FAMILY WHICH CONTAIN AN ENHANCED PHOB OR PHOR GENE



(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the fol-  
lowing steps are carried out: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino  
acid and in which at least one or more of the genes chosen from the group consisting of phoB and phoR, or nucleotide sequences  
which code for these, is or are enhanced, in particular over-expressed, b) concentration of the desired L-amino acid in the medium  
or in the cells of the bacteria, and c) isolation of the desired L-amino acid.

WO 03/008606 A3

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 02/07355

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7 C12N15/31 C12P13/08 C12P13/04 C07K14/245 //(C12P13/08, C12R1:19)		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N C12P C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
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-/--		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family		
Date of the actual completion of the international search  16 October 2003		Date of mailing of the international search report  28 OCT 2003
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer  van de Kamp, M

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/07355

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	WO 99 53035 A (ALTMAN ELLIOT ;GOKARN RAVI R (US); EITEMAN MARK A (US); UNIV GEORG) 21 October 1999 (1999-10-21) page 5, line 20-24 examples 4,7,9,10 claims 31,38,41,49 figures 1,4 ---	1-7
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A	EP 0 237 819 A (KYOWA HAKKO KOGYO KK) 23 September 1987 (1987-09-23) the whole document ---	1-7
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## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 02/07355

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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E	<p>WO 03 008605 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 30 January 2003 (2003-01-30) the whole document claims 1,6</p> <p>---</p>	1-7
E	<p>WO 03 008607 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 30 January 2003 (2003-01-30) the whole document claims 1,6</p> <p>---</p>	1-7
E	<p>WO 03 008608 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 30 January 2003 (2003-01-30) the whole document claims 1,6</p> <p>---</p>	1-7
E	<p>WO 03 008609 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 30 January 2003 (2003-01-30) the whole document claims 1,6</p> <p>---</p>	1-7
E	<p>WO 03 008610 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 30 January 2003 (2003-01-30) the whole document claims 1,6</p> <p>---</p>	1-7
E	<p>WO 03 008611 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 30 January 2003 (2003-01-30) the whole document claims 1,6</p> <p>---</p>	1-7
E	<p>WO 03 008612 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 30 January 2003 (2003-01-30) the whole document claims 1,6</p> <p>---</p>	1-7
E	<p>WO 03 008613 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 30 January 2003 (2003-01-30) the whole document claims 1,6</p> <p>---</p>	1-7
E	<p>WO 03 008614 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 30 January 2003 (2003-01-30) the whole document claims 1,6</p> <p>---</p>	1-7
	<p>---</p> <p>-/--</p>	

## INTERNATIONAL SEARCH REPORT

Internat Application No  
PCT/EP 02/07355

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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T	WO 03 008600 A (DEGUSSA ;HERMANN THOMAS (DE)) 30 January 2003 (2003-01-30) the whole document claim 1 -----	1-7

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 02/07355

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-7 (all partially)

A process for the preparation of L-amino acids, in particular L-threonine, comprising the steps of a) fermenting a microorganism of the Enterobacteriaceae family which produces the desired L-amino acid and in which the *phoB* gene is enhanced, in particular overexpressed, b) concentrating and (c) isolating the desired L-amino acid, as well as a process as said in which additional genes are enhanced and/or attenuated.

2. Claims: 1-7 (all partially)

A process for the preparation of L-amino acids, in particular L-threonine, comprising the steps of a) fermenting a microorganism of the Enterobacteriaceae family which produces the desired L-amino acid and in which the *phoR* gene is enhanced, in particular overexpressed, b) concentrating and (c) isolating the desired L-amino acid, as well as a process as said in which additional genes are enhanced and/or attenuated.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/07355

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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